IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Zyskind, J. W., et al. Art Unit : 1655
Serial No. : 08/971,090 Examiner : Jeffrey Fredman, Ph.D.
Filed : November 14, 1997
Title : METHOD FOR IDENTIFYING MICROBIAL PROLIFERATION GENES

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132

Sir:

1. I, , Dr. Robert W. Simons, Ph.D., am an Associate Professor in the Department of Microbiology, Immunology and Molecular Genetics, 1602 Molecular Science, University of California, Los Angeles, CA 90095.

2. I am an expert in the general fields of molecular biology and molecular genetics, as well as the specific field of antisense RNA control. I was considered an expert in these fields in 1997 at the time of the invention. I am one of the co-discoverers of natural antisense RNA control of gene expression.[1] I have conducted research and published numerous research articles, reviews and book chapters on antisense RNA control. My curriculum vitae is attached as documentation of my credentials.

3. Since 1985, I have consulted with several companies and many research colleagues on the development and use of artificial antisense RNA control. Currently, I have a consulting arrangement with Elitra Pharmaceuticals, Inc., 3510 Dunhill Street, Suite A, San Diego, CA 92121, the exclusive licensee of the above-identified patent application (hereinafter referred to as "Elitra").

4. I have read the specification and the file history, including past and outstanding office actions, and Applicants' responses, for the above-referenced patent application. I understand the issues presented by the Patent Office in the outstanding office action regarding pending claims of the application (which will be referred to hereinafter as "the

[1] See, e.g., Simons, et al., (1988) *Annu. Rev. Genet.* 22:567-600

invention"). It is my understanding that the Patent Office rejected all pending claims as allegedly obvious over Spann in view of either Cormier-Regard or Timberlake and further in view of Gossen. The Patent Office had alleged that Spann is deficient in that "Spann does not teach application of the method [of the invention] to genes involved in microbial proliferation" and that "Spann also does not teach the range of organisms claimed." Applicants argued that Spann is further deficient because it does not teach use of random fragments, and, neither Cormier-Regard, Timberlake, nor Gossen cure this deficiency. In response, the Patent Office modified this rejection by adding Escher to cure this deficiency in Spann. Accordingly, one issue is whether there was any suggestion or teaching in the art to combine Escher with Spann.

5. It is my opinion that there is no express or implied suggestion in Escher to combine that teaching with Spann and that there is no express or implied teaching in Spann to combine that teaching with Escher. Escher's goal was to find new functional fragments of genomic DNA that could activate, or "turn on," transcription using an assay called a "[transcriptional] activator trap" method. Escher's method needed a source of random fragments of genomic DNA to insert into reporter constructs used in the activator trap assay. Sonicated DNA was used as that source. Escher made no suggestion that this source of DNA, or the use of random fragments of genomic DNA, could or should be used in other assays, e.g., assays like Spann's that express antisense cDNAs to identify genes necessary in development. Furthermore, Spann, using antisense cDNA, does not recognize that his method has deficiencies and does not suggest or recognize a need for an alternative source of nucleic acid to identify genes necessary in development.

6. Furthermore, it is my opinion that nowhere in the prior art, as of 1997, at the time of the invention, was there any suggestion or teaching in the art to combine methods, or sources of DNA, as used in transcriptional activator trap assays (as described by Escher) with assays for expressing different antisense cDNAs to identify genes necessary in development (as described by Spann).

7. Moreover, it is my opinion that, at the time of the invention, in 1997, based on either a general, fundamental understanding of these two disparate arts,[2] or, based on a specific reading of Eschar or Spann, neither I, as an artisan skilled in these arts, nor another skilled artisan, would have been motivated to combine teachings from these two disparate arts, or, specifically, to combine Eschar and Spann. While in 1997 I was fully aware of the basic principles underlying each of these components, I nevertheless did not routinely look to the transcriptional regulatory literature to keep up to date with my specific field of research (on antisense RNA control), nor did I look to that field as a source of new methodologies. Despite my research activities on antisense RNA control at the time of the invention, which occurred in the midst of numerous discussions on artificial antisense RNA control with other highly knowledgeable experts in this field, neither I, nor to my knowledge any of my colleagues, was able to conceive or suggest the invention. Accordingly, at the time of the invention I did not and I do not believe a skilled artisan would have been motivated to combine Spann and Eschar.

8. At the time of the invention, in 1997, I and my colleagues, skilled artisans in the field of artificial antisense RNA control, were skeptical that there was a way to readily identify artificial antisense sequences.[3,4] At the time of its discovery in 1985, antisense RNA control of gene expression was wrongly viewed as simple because it only required the completely predictable Watson-and-Crick complementarity between sense (natural message) and antisense nucleic acids. However, by 1997, at the time of the invention, it was recognized, as reflected in the prior art, that the lessons emerging from studies on natural antisense RNA control could not be easily applied to artificial antisense cases, despite considerable effort. Diverse

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[2] The disparate arts involved are: transcriptional activation (including transcriptional activator trap assays); and, use of antisense cDNA to identify genes necessary in development (including assays for expressing different antisense cDNAs).

[3] In one embodiment, the invention readily identifies effective antisense sequences (see explanation, below) to identify microbial proliferation genes. Thus, such skepticism "taught away" from the use of antisense sequences to find a means to readily identify essential microbial proliferation genes.

[4] In one embodiment of the invention, an essential gene is identified when translation of its message into protein is inhibited by its binding to an antisense sequence (and lack of or inappropriate expression of the protein determines if the gene is essential). An antisense sequence is a nucleic acid having a sequence complementary to a portion of a natural message – because only a few segments of the message are amenable to inhibition by binding to an antisense, and because there were no paradigms to predict which segments, when bound to antisense, block message expression, it was the inability to readily identify such segments that confounded the field at the time of the invention.

strategies for screening for effective (inhibitory) antisense constructs had largely failed. Indeed, by 1997, the prior art reflects extreme skepticism, even pessimism, that there was any means that could predictably screen for message-inhibiting antisense. This skepticism was reflected in the literature at the time of the invention. A typical publication reflecting the skepticism of the times can be found in, e.g., Stein, C.A. "Does antisense exist ? It may, but only under very special circumstances" (1995) *Nature Medicine* **1**:1119-1121.[5] This paper described and reviewed problems being encountered with antisense at the time of the invention. In addition to discussing difficulties related to inherent biochemical properties of nucleic acids that made developing antisense therapeutic drug molecules problematic, papers of the time also addressed the issue of whether it was possible to make effective antisense molecules based on the assumption that simple Watson-Crick complementarity was essentially all that was required. The skilled artisans clearly concluded, and with some degree of frustration, that more than simple Watson-Crick complementarity was needed. They also concluded that it was not at all clear how to solve this problem. Efforts to find an effective paradigm for designing an effective antisense met with failure. Screening randomly chosen antisense oligonucleotides more often met with failure than success. Neither analyses of these results nor investigations into the actions of naturally occurring antisense RNAs provided any clear rules about how to design effective antisense molecules. Indeed, Stein (1995) concludes "...it is not unreasonable to suspect that sequence-specific inhibition of gene expression, at least with these constructs, does not exist at all." Another typical publication reflecting the skepticism of the times can be found in, e.g., Branch, A.D. "A good antisense molecule is hard to find" (1998) *TIBS* **23**:45-50. This paper, which appeared early in 1998, describes problems being encountered at the time of the invention. In my opinion, Branch is even more pessimistic than Stein. In particular, it describes how "the antisense field has been turned on its head by the discovery of 'non-antisense' effects..." and teaches that "[antisense molecules] are far more difficult to produce than was originally anticipated." Art published in *TIBS* is widely read. Thus, the extreme skepticism of the literature at the time of the invention about the use of artificial antisense RNA control, in

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[5] This skepticism in the art remained for many years, see, e.g., the exemplary reviews: Flanagan, W.M. "Antisense comes of age" (1998) *Cancer and Metastasis Reviews* **17**:169-176; Wallace, R.W. "Does antisense make sense?" (1999) *Drug Discovery Today* **4**:4-5).

combination with the many published failed attempts at finding a general paradigm for identifying effective antisense, "taught away" from the idea that genes necessary for proliferation could be readily identified, particularly by use of artificial antisense RNA control. At the time of the invention, one practiced in the art would almost certainly have encountered this skepticism and "teaching away" very early in developing strategies for identifying antisense sequences.

9. At the time of the invention, in 1997, I was severely skeptical that it was possible to design rapid and predictable screens for identifying inhibitory artificial antisense RNA (and, thus, identify an essential gene). I was severely skeptical that artificial antisense RNA could be engineered in a systematic way. I gave this opinion as advice to colleagues and other research scientists. Thus, after initiating my consulting arrangement with Elitra and learning of the invention and the method's success in the hands of Elitra scientists, I was considerably surprised.[6] Indeed, based on nearly 20 years of my own research and thought, reinforced by a state of the art that "taught away" from such a concept, I initially remained skeptical. However, the invention's continued success at rapidly facilitating the identification of essential genes in microorganisms has unambiguously proven its utility and value.

10. Thus, whereas the individual elements of the invention may have existed as discrete elements in disparate arts at the time of the invention, I do not believe that the unique combination of elements that constitutes the invention was suggested, or obvious, at that time. Indeed, given the intense desire within the field for effective and specific artificial antisense RNA strategies, and the large number of individuals involved in that quest prior to 1997, had the combination been suggested, or obvious, I believe it would have been tried several times over. Moreover, I believe that the numerous failures of diverse antisense RNA strategies, as reflected in the prior art as of 1997, rather than motivating the sort of innovative combination of elements that constitutes the invention, actually dissuaded and taught away from such innovation.

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[6] The success of the claimed method (in contrast to failures of the prior art) is due, in part, to use of large libraries of random genomic nucleic acid fragments, in which (surprisingly) there is almost always a fragment that will be inhibitory.

I hereby declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully Submitted

Date: August 7, 2000

Robert W. Simons

APPENDIX A
TO
DECLARATION OF ROBERT W. SIMONS

Does antisense exist?

It may, but only under very special circumstances.

C.A. STEIN

At the present time, for better or worse, antisense oligodeoxynucleotide technology is heavily dependent on phosphorothioate oligodeoxynucleotides. It should be recalled that the reason that phosphorothioate oligodeoxynucleotides were originally synthesized was because of the nuclease-sensitivity of phosphodiester oligodeoxynucleotides. The latter compounds appear to be digested in a 3'–5' direction, and thus are unsuitable for use as therapeutic agents. In contrast, the phosphorothioate oligomers are highly nuclease resistant (although not nuclease-proof). The substitution of sulphur at each phosphorus is conservative and results in an oligomer that hybridizes with reasonably high T_m to its mRNA complement. Phosphorothioates can also elicit RNase H activity as a DNA-mRNA complex (RNase H is a ubiquitous enzyme that cleaves the mRNA strand of this duplex. However, phosphorothioates can also, at higher concentration, bind directly to RNase H and inhibit its catalytic action[1]. Furthermore, even though the sulphur-for-oxygen substitution generates a center of chirality at phosphorus, it is unclear to what extent, if any, this potentially unfavourable property is biologically significant. In addition, the pharmacokinetics of phosphorothioate oligodeoxynucleotides appear to be generally favourable for their development into a therapeutic agent. Nevertheless, despite all these advantages, it is extraordinarily difficult to demonstrate that the biological effects observed in a large number of experiments performed over the years, mostly in tissue culture, are due entirely to sequence-specific inhibition of genetic expression by phosphorothioate oligodeoxynucleotides.

Phosphorothioate oligodeoxynucleotides, as we now know, are fundamentally different compounds than their phosphodiester congeners, although earlier this might have been difficult to fully appreciate. Dubbed 'informational drugs', it now appears that these compounds contain a surfeit of information, much of it unrelated to Watson–Crick base pair hybridization.

Phosphorothioate oligodeoxynucleotides are polyanions, and as such are capable of binding the same proteins that other polyanions, especially heparin, also bind. This binding is primarily based on a charge interaction, but more recent evidence (discussed below) indicates that the binding may depend in part on base sequence as well. (When base sequence becomes important, the oligomer is known as an 'aptamer'). I have determined that the dissociation constant (K_d) for a phosphorothioate-protein complex tends to be about one to three orders of magnitude lower than that for an equal-length phosphodiester oligodeoxynucleotide. The reasons for this are, at present, imperfectly understood, but some evidence exists that a major contributor is the very slow dissociation rate of the phosphorothioate-protein complex.

The non-sequence-specific interaction of phosphorothioate oligodeoxynucleotides with proteins and/or nucleic acid targets may cause interesting — and potentially useful — biological effects. For example, phosphorothioates, in a length-dependent but relatively sequence-independent manner, bind to recombinant soluble CD4 at or near the HIV-1 binding site[2], and also bind to the v3 loop of the HIV-1 envelope glycoprotein, gp120 (ref. 3). Both of these nonspecific interactions can, depending on the test system employed, be useful inhibitors of HIV-1 infectivity and cytopathogenicity. Moreover, even so-called sequence-specific phosphorothioates, if sufficiently long and if used at sufficiently high concentration, can exhibit these non-sequence-specific but potentially therapeutic effects. The net result in this system is to make it quite difficult for the experimenter to determine precisely which observed effects are antisense in nature, and which are caused by a complex mix of sequence specific plus non-sequence-specific effects.

This situation becomes far more intricate when the complexities of mammalian cells are considered. For example, the ability of phosphorothioates to bind to heparin-binding proteins may become critical determinants of their biological behaviour. Guvakova *et al.*[4] have recently shown that phosphorothioates, in a length- and concentration-dependent manner, can directly bind to basic fibroblast growth factor (bFGF), acidic FGF, FGF-4, platelet-derived growth factor and vascular endothelial growth factor. In the case of bFGF, the binding can lead to blockade of binding of bFGF to both its low- and high-affinity receptors. Phosphorothioate oligomers can also remove bFGF from low-affinity storage sites in extracellular matrix. Phosphorothioates in these respects resemble the polyanions suramin and pentosan polysulphate, two compounds that are currently in clinical cancer trials. In contrast, phosphorothioates do not appear to directly bind to epidermal growth factor, which itself weakly binds heparin, if at all.

These observations are important in light of recent experiments performed by Simons *et al.*[5]. They examined the rat carotid model of restenosis following balloon angioplasty injury. A phosphorothioate oligomer targeted to the c-*myb* oncogene was applied to the adventitia of the carotid in a pluronic gel. Sequence-specific inhibition of restenosis was claimed, but it now appears that this result is predominately non-sequence-specific[6]. I believe that it is entirely possible that non-sequence-specific inhibition of the binding of heparin-binding growth factors to their receptors and/or removal of bFGF from low-affinity binding sites on extracellular matrix by the phosphorothioate oligomer are some of the root causes of the effects observed by Simons *et al.*

It could be asked how thoughtful, careful scientists could possibly be bamboozled? If the antisense oligomer produces a biological effect, and the sense oligomer does not, is that not indeed *prima facia* evidence for sequence-specificity? Unfortunately, when phosphorothioate oligodeoxynucleotides are used, bamboozlement hardly seems to be the exception. A further layer of complexity is added on when it is appreciated that the binding of the phosphorothioates to bFGF is highly sequence-dependent, especially when the oligomer contains four contiguous guanosine residues (the infamous 'G-quartet'). It is not clear why the G-quar-

tet contributes to increase affinity for protein, but I speculate that the answer relates to the phenomenon of non-Watson–Crick binding between the adjacent guanosines. This interaction may reduce the number of degrees of rotational freedom of the oligomer relative to a non-G-quartet-containing compound. This reduction may in turn favour the binding of the oligomer to protein, initially, via a primary charge interaction (as described above). The final state, in which the oligomer is protein-bound, can be viewed as one in which the oligomer has suffered an extreme loss of rotational freedom. Regardless of the mechanism, the presence of the G-quartet motif within the sequence of a phosphorothioate oligodeoxynucleotide can create havoc with the notion of sequence-specific inhibition of genetic expression.

Another dramatic example of such havoc was demonstrated recently by Khaled *et al.* (personal communication) They determined that laminin, a heparin-binding protein, also (and unsurprisingly) interacts with phosphorothioate oligodeoxynucleotides. One of the effects of this interaction is to block the binding of laminin to its ligand, galactosyl ceramide sulphate (sulphatide), a ligand in the extracellular matrix on which cells can spread and grow. Via this mechanism, and several others as well, phosphorothioates can be profoundly anti-adhesive. However, the ability of phosphorothioates to block the binding of laminin to sulphatide was heavily dependent on the presence of a G-quartet; in fact, the IC_{50} of inhibition favoured the G-quartet oligomer over a control oligomer by a factor of 40. In this case, the G-quartet-containing oligomer was actually targeted to the p65 (Rel A) subunit of NF-κB, a nuclear transcriptional regulatory factor, whereas the control (sense) construct *by definition* did not contain the G-quartet. Thus, it can be extremely difficult to determine if the antisense Rel A phosphorothioate oligomer is anti-adhesive because of an antisense mechanism, as has been suggested, or because of a sequence-selective, non-antisense mechanism based (at least partially) on inhibition of the binding of laminin to sulphatide. Similar problems will most likely arise in any laminin-dependent assay system in which phosphorothioate oligodeoxynucleotides are employed as 'antisense' reagents (for example, see ref. 7).

But if we know that the G-quartet motif produces problems in data interpretation, cannot we utilize appropriate phosphorothioate oligomer controls to take account of this? The answer is only maybe, and then only if luck prevails. This paradoxical situation exists primarily because all phosphorothioate oligomers containing G-quartets are not created equal (as demonstrated by Maltese *et al.*[8]). These authors examined the non-sequence-specific inhibition of cellular adhesion, suppression of nuclear Sp1 transcription factor activity and blockade of cytoplasmic to nuclear NF-κB translocation by a series of phosphorothioate oligodeoxynucleotides. They concluded that the mere presence of a G-quartet in the oligomer does not necessarily result in excessive non-sequence specificity. Rather, it is the presence of the G-quartet plus the flanking sequences that are responsible for the nonspecific effects labelled as originating in the G-quartet. However, the authors could not discern any general rule to specify which flanking sequences were responsible for augmenting the non-sequence specificity of the G-quartet motif. Thus, if this problem is a general one (as it seems to be) it thus logically becomes virtually impossible to produce an appropriate control phosphorothioate oligomer sequence when the G-quartet motif is present in the antisense construct. And if this is true (and the evidence to date indicates that it is), then how can data obtained from an experiment in which the G-quartet is present be unambiguously interpreted as resulting from a sequence-specific antisense effect?

But (it could be argued) it is also self-evident that not all antisense phosphorothioate oligodeoxynucleotides must contain the G-quartet motif. If this specific motif is avoided, cannot antisense sequence specificity be unambiguously demonstrated? The answer again is 'no', unfortunately, and this time the ambiguity may actually be most pronounced *in vivo*. This is because phosphorothioate oligodeoxynucleotides containing the simple CpG motif may be profoundly immunemodulating. For example, they stimulate mouse spleen cells; when injected intraperitoneally into mice, phosphorothioates can stimulate a dramatic increase in immunoglobulin secretion within 24 hours and increase expression of activation markers such as MHC class II (ref. 9). Furthermore, phosphorothioate oligomers containing the CpG motif may induce interfer-

Those who would attempt antisense work could be entering a heavenly garden of scientific delight ...

ons[10], and may also augment NK cell activity[11]. More recent evidence (A. Krieg, personal communication) suggests that CpG-containing phosphorothioates may also modulate T-cell function, and may stimulate release of several members of the interleukin family. The effects of such immune modulation must inevitably call into some question the interpretation of results obtained in murine tumour models, although it is not certain what, if any, effect the CpG motif has in humans. Once again the question arises as to the proper control sequences that should be employed in these experiments, as the sequence context in which the CpG motif occurs almost certainly is a matter of some importance. Moreover, it is not clear that the CpG motif is the only one that will generate unusual — and always unpredictable — biological effects. In my opinion, it is likely that additional motifs will eventually be discovered that induce novel cellular behaviour. All of these effects are fascinating in their own right, are worthy of intense study, and may eventually be elaborated into a therapeutic modality. But they definitely ain't antisense.

Given all of the problems associated with interpretation of data derived from experiments performed with phosphorothioate oligodeoxynucleotides (see ref. 12 for some general guidelines about this problem), it is not unreasonable to suspect that sequence-specific inhibition of gene expression, at least with these constructs, does not exist at all. However, I do not believe that this suspicion is correct. Although there is no doubt that great caution must be exercised in the evaluation of data obtained with phosphorothioates if a claim of sequence specificity is desired, it is also becoming clear that if the oligomer concentration is kept low (probably <5 μM, and ideally <1 μM), the majority of the non-sequence specific effects may be severely vitiated. This is also true if the length of the oligomer is kept short (no longer than 15–17 bases). Phosphorothioates containing known trouble-making motifs, such as the G-quartet or CpG, should be handled with special care, when they are handled at all. More than one control oligomer should always be employed in addition to the antisense construct[12]. In general, it is probably true that the more controls that are used, the better the chance that the observed end point is truly reflective of an antisense mechanism. The observed biological end point should virtually always include a demonstration of diminution in concentration of the protein product of the targeted mRNA. Finally, seemingly unusual experimental results should not merely be discarded in favour of reporting those that were initially desired. As demonstrated above, it is not uncommon that the non-sequence specific effects of oligodeoxynucleotides are just as interesting, and potentially just as therapeutically useful, as those that have been labeled sequence-specific.

... or otherwise. (Paintings by H. Bosch, 16th century.)

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Columbia University
College of Physicians and Surgeons
New York, New York 10032, USA

the folding of the protease sequences when added as separate molecules, both *in vitro* and *in vivo*[15]. One way in which cells change the quantitative properties of proteins is to make allosteric effectors; this method is reversible and requires the continual presence of the effector. Perhaps another method useful in say, terminal differentiation, is the production of separate steric chaperones that irreversibly change the properties of certain specific proteins by influencing their folding.

Acknowledgements

I thank Chris Dobson and Ulrich Hartl for commenting on this article, and Sheena Radford and Nick Price for advice on specific points.

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R. JOHN ELLIS

Department of Biological Sciences, University of Warwick, Coventry, UK CV4 7AL.
Email: je@dna.bio.warwick.ac.uk

A good antisense molecule is hard to find

Andrea D. Branch

Antisense molecules and ribozymes capture the imagination with their promise of rational drug design and exquisite specificity. However, they are far more difficult to produce than was originally anticipated, and their ability to eliminate the function of a single gene has never been proven. Furthermore, a wide variety of unexpected non-antisense effects have come to light. Although some of these side effects will almost certainly have clinical value, they make it hard to produce drugs that act primarily through true antisense mechanisms and complicate the use of antisense compounds as research reagents. To minimize unwanted non-antisense effects, investigators are searching for antisense compounds and ribozymes whose target sites are particularly vulnerable to attack. This is a challenging quest.

ANTISENSE STRATEGIES LOOK almost too easy on paper. Simple and elegant schemes can be drawn for both antisense oligodeoxynucleotides (ODNs – short DNA molecules intended to bind to and inhibit target RNAs through complementary Watson–Crick base pairing) and bioengineered ribozymes (catalytic RNA molecules intended to bind and cleave target RNAs). Scientists seek to use these molecules to ablate selected genes and thereby understand their functions, and pharmaceutical developers are working to find nucleic-acid-based therapies. However, the antisense field has been turned on its head by the discovery of 'non-antisense' effects, which occur when a nucleic acid drug acts on some molecule other than its intended target – often through an entirely unexpected mechanism. Non-antisense effects are not necessarily bad. Indeed, some may prove to be a boon to the pharmaceutical industry because they offer an added source of potency. However, their unpredictability confounds research applications of nucleic acid reagents.

Non-antisense effects are not the only impediments to rational antisense drug design. The internal structures of target RNAs and their associations with cellular proteins create physical barriers, which render most potential binding sites inaccessible to antisense molecules. For Watson–Crick base pairing to occur, nucleic acid drugs must be complementary to exposed regions in their target RNAs and must co-localize with them. When these requirements are met, true antisense effects are enhanced, and unwanted non-antisense effects are minimized. However, optimization is a time-consuming process. Currently, effective nucleic acid drugs must be selected from large pools of candidates. Streamlined approaches for (irrational) *in vivo* selection are needed to speed the discovery of active molecules.

A. D. Branch is in the Division of Liver Diseases, Box 1633, Department of Medicine, The Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA.

Non-antisense effects: quicksand for some, diamond mines for others

The potential of nucleic acid drugs to deliver 'exquisite specificity'[1] is frequently cited: antisense methods are credited with offering 'the specificity of the genetic code and the versatility of targeting any number of proteins'[2]; and it is said that a therapeutic ribozyme 'can be designed to interact only with its target, and the target is expected to appear only once in the genome, giving one a high degree of assurance that the target – and only that target – has been inhibited'[3]. However, it has never been proven that antisense drugs have the capacity to knock out just one gene, although both ODNs and bioengineered ribozymes can undeniably hit their intended targets[4,5]. The powerful appeal of antisense strategies has been a mixed blessing. The twin concepts that effective antisense reagents are easy to

 0968-0004/98/$19.00 PII: S0968-0004(97)01155-9

design and that they selectively home in on their targets have overshadowed the cautionary messages in articles such as 'Antisense has growing pains'[6], 'Can hammerhead ribozymes be efficient tools to inactivate gene function?'[7], and 'Does antisense exist? (It may, but only under very special circumstances)'[8].

The purpose of this article is to review the factors that make and break specificity in antisense applications and to discuss the need to judge therapeutic compounds and research reagents by separate standards. Only antisense molecules[9–11] and ribozymes[12,13], designed to inhibit RNA targets are considered here, but many of the principles apply to other nucleic acid drugs, such as those used to correct DNA mutations[14], to alter RNA splicing[15], and to control gene expression by forming triple helices with DNA (Ref. 16).

Non-antisense effects pose a dilemma for the pharmaceutical industry[17]. These effects include the stimulation of B-cell proliferation[18] and the inhibition of viral entry into cells[19], responses which are potentially useful. Non-antisense ODNs are already being developed as adjuvants to boost the efficacy of immunotherapies and vaccines[20]. Phase III clinical trials of ISIS 2922 (Ref. 21), a phosphorothioate oligonucleotide (S-ODN) that induces both antisense and non-antisense effects, are also under way in patients with cytomegalovirus-associated retinitis[22]. It is hoped that this compound's diverse mechanisms of action will yield a single drug that provides many of the benefits of combination therapy. However, as Anderson and colleagues have observed, characteristics that are advantages in pharmaceutical drugs can be disadvantages in research reagents[21]. Thus, a safe and effective nucleic acid drug that slows the progression of AIDS would be of tremendous value, even if it were to act by inhibiting a perplexing combination of viral proteins rather than by binding to HIV RNA as originally intended. However, this same compound would be useless as an agent to selectively destroy HIV RNA, and could be ruinous if used in experiments of HIV molecular biology without knowledge of its mechanism of action. Because a single, well-understood mechanism of action cannot be assumed, non-antisense effects create major difficulties for gene hunters. Years of investigation can be required to figure out what an 'antisense' molecule is actually doing, as discussed further below.

Non-antisense effects also have a downside for pharmaceutical developers. Because knowledge of their underlying mechanisms is typically lacking, non-antisense effects muddy the waters. They make true antisense drugs more difficult to design and harder to commercialize. Furthermore, they can be a source of toxicity.

All drugs are dirty: clinical benefit is the pharmaceutical gold standard

Stanley Crooke (Isis Pharmaceuticals) stresses that 'a vast body of experience says that no drug is entirely selective'[23]. Because biologically active compounds generally have a variety of effects, dose–response curves are always needed to establish a compound's primary pharmacological identity. Antisense compounds are no exception. As is true of all pharmaceuticals, the value of a potential antisense drug can only be judged after its intended clinical use is known, and quantitative information about its dose–response curves and therapeutic index is available.

It may be surprising to hear antisense molecules described in the same terms as conventional drugs, but, in fact, nucleic acid drugs should not be thought of as magic bullets. Their therapeutic use will require vigilant monitoring. Compared to the dose–response curves of conventional drugs, which typically span two to three orders of magnitude, those of antisense drugs extend only across a narrow concentration range. Both *in vitro* and *in vivo*, less than a factor of ten often separates the concentration producing no antisense effect from that producing the full antisense effect[22]. Steep dose–response curves commonly indicate that a drug has multiple, synergistic mechanisms of action[24]. A drug with a narrow therapeutic window can be potent and extremely valuable, but can also be tricky to use safely. Since the ratio of antisense to non-antisense effects drops sharply outside a restricted concentration range, it will be challenging to obtain consistent therapeutic results.

Mother Nature's cruel antisense jokes lead to tougher experimental standards

Their powerful allure and favorable press have often caused the problems associated with antisense reagents to be trivialized. In some cases, relaxed standards have been applied. Arthur Krieg (University of Iowa) provided insight into the need for stricter quality control when he shared the results of an informal poll. He reported that 'the estimate that many people have given me of the percentage of accurate published antisense papers ranges from 50% of them being accurate to 5% being accurate'[22].

As discussed previously, when an antisense molecule causes a biological effect, it can be extremely difficult to determine whether the change occurred because the reagent interacted specifically with its target RNA, or because some non-antisense reaction – involving other nucleic acids or proteins – was set in motion[8,25]. When attempting to distinguish between antisense and non-antisense effects, a common strategy has been to use an oligonucleotide in which the sequence of the antisense oligonucleotide is altered. Unfortunately, not all non-antisense effects can be readily detected by this approach, as illustrated by studies of antisense therapies for chronic myeloid leukemia. In this disease, a chromosomal translocation often produces the Philadelphia chromosome, resulting in the synthesis of an oncogenic fusion protein, BCR/ABL. The mRNA for this protein has been regarded as an ideal target for antisense therapies. Several groups have reported inhibition of leukemic cell proliferation by anti-BCR/ABL antisense oligonucleotides. In fact, Vaerman and co-workers cite 16 publications reporting promising findings[26]. However, they discovered that a disappointing, non-antisense mechanism was responsible for their own results, adding weight to studies showing that S-ODNs block proliferation through non-antisense mechanisms (reviewed in Ref. 26). Recent work indicates that cytotoxic ODN breakdown products are responsible for the antiproliferative effects observed[27]. These studies strongly underscore the need to test numerous control ODNs when carrying out antisense research, and to maintain a high index of suspicion.

C. A. Stein (Columbia University) has reviewed many 'non-sequence-specific' (non-antisense) effects caused by S-ODNs, providing dramatic examples of the havoc that has resulted when S-ODNs have unleashed their surfeit of cryptic information. S-ODNs are used because their modified backbones confer nuclease resistance. However, they bind avidly to many proteins, forming complexes with dissociation constants one to three orders of magnitude lower than those of phosphodiester ODNs. In a test of B-cell proliferation and differentiation, S-ODNs were two logs more potent than phosphodiester ODNs of the same sequence[28]. According to Stein, S-ODNs have 'bamboozled' many researchers by inducing biological effects that mimic, and are mistaken for, true and desired antisense effects[8,19].

Addressing the manifest need for stricter experimental standards, Arthur Krieg and C. A. Stein (editors of the journal *Antisense and Nucleic Acid Drug Development*) have published guidelines for designing antisense studies[1]. Recently, the need to use pure oligonucleotide reagents has been stressed. The selective publication of expected (positive) results is being actively discouraged. The confusion that has thus far occurred indicates that each new 'antisense' molecule needs to be tested exhaustively.

How close do current antisense techniques come to single-gene accuracy?

While the ability to knock out a single gene is a luxury in a pharmaceutical compound, specificity is a key feature of a reagent to be used in a research setting. Although single-gene accuracy is not essential for an experimental reagent to be useful, the extraneous perturbations it causes need to be identified. Additionally, as alternative approaches for selective gene ablation (such as the production of genetic knockouts) improve and become easier to carry out, it will be important to know how antisense techniques compare in terms of time, expense and selectivity. This comparison awaits additional information about antisense specificity.

Unfortunately, quantitative data about the magnitude of antisense-induced side reactions are limited. Most of the information is extrapolated from experiments in which the impact of an antisense compound is measured on only a small number of molecules: the intended target RNA, a housekeeping gene, and perhaps a few control RNAs. An antisense molecule is typically taken to be 'specific' if two criteria are met: (1) there is no gross loss of cell viability, and (2) the levels of the target RNA and its associated protein fall much more than those of the control RNAs. However, this type of experimental design is too limited in scope to provide information about global changes in the RNA and protein populations. It does not provide even a rough measure of the signal-to-total noise ratio. Unlike the analysis of Scatchard plots, which allows the interactions between a ligand and a complex mixture of proteins to be explored, this design looks at three or four RNAs and projects the impact on the remaining 10^5 genes. As an additional shortcoming, it provides no direct information about interactions between the antisense molecule and proteins, even though these interactions may lead to the major effects caused by 'antisense' molecules. Because it could provide a before-and-after snap-shot of the protein population, high-resolution two-dimensional gel electrophoresis[29] might shed light on the spectrum of changes induced by antisense molecules. However, a recent round-table discussion suggested that there are no published studies in which this technique has been utilized to evaluate antisense specificity[22].

So far, the concept that an antisense molecule can selectively knock out a single gene appears to have been untested. In the future, several techniques, in addition to two-dimensional gel electrophoresis, might be employed to investigate antisense specificity. For example, as the repository of sequenced genes grows, it will be possible to identify RNAs that contain regions complementary to an antisense molecule and to measure the impact of antisense treatments on these bystander molecules. In addition, broad surveys of mRNA populations could be conducted. To identify changes induced by antisense treatments, RNA from treated and control cells could be reverse-transcribed and the resulting cDNA populations analyzed either by differential display, which separates cDNAs electrophoretically, or by hybridization to gene chips, which are being developed to allow the quantitative monitoring of gene expression patterns[30]. Should unanticipated changes be detected by such surveys, other techniques could be used to distinguish those caused by lack of specificity from those reflecting downstream consequences of the intended antisense reaction. Information about the number of accidental hits and about the nature of the interactions responsible for the changes in the expression of other genes would be useful and would guide future drug development. Today's peak specificity, whatever it is, will almost certainly rise as current strategies are optimized and advances in nucleic acid chemistry bring derivatives with fewer side effects. New compounds are currently under investigation[17,31] and additional derivatives can be expected in the future.

Theoretical limits of specificity

Theoretical calculations provide a useful perspective on antisense specificity. The haploid human genome contains about 3×10^9 bases. In a *random* sequence of this size, any sequence that is 17 nucleotides long or longer would have a high probability of occurring only once – of being unique. To knock out a single gene, an intervention would have to distinguish a 17-base perfect match from one with a single-base mismatch.

In considering whether ODNs have the requisite power of discrimination, it is crucial to know their mechanism(s) of action. These mechanisms may differ from cell type to cell type and may depend upon the exact nature of the target RNA and the ODN. However, there is strong evidence that in several systems, including *Xenopus* oocytes[32] and permeabilized cells[33], the target RNA is destroyed by the action of RNase H. RNase H activities cleave the RNA component of DNA–RNA hybrids. They do not require long hybrid regions as substrates. In fact, *in vitro*, RNase H can cleave a hybrid containing only 4 bp (Ref. 34). In *Xenopus* oocytes, as few as 10 bp are sufficient[35]. For standard ODNs, it is likely that 10 bp are also sufficient in human cells; in the case of certain chemically modified nucleotides, it is proven that as few as 7 bp can lead to cleavage[36]. Random sequences the length of the human genome contain an average of 3000 copies of each 10-nucleotide sequence (10-mer). Thus, it is extremely likely that any particular 10-mer will occur in many RNAs. When an ODN complementary to this 10-mer is introduced into a cell, all of the RNAs containing this 10-mer are at risk for RNase H-mediated cleavage. Of course, not all 3000 copies will be susceptible to cleavage: many will not be present in transcripts, and many that are present in transcripts will be inaccessible. However, if even 1% of the 3000 are hit, 30 genes will be directly affected. Furthermore, the number of 'at risk' sites is probably more than an order of magnitude greater than 3000 for two reasons: (1) ODNs typically contain 20 or more bases, each 20-mer contains 11 10-mers, and each 10-mer would be present 3000 times, on average; and (2) in all likelihood, RNase H does not require 10 consecutive bp for cleavage. Because RNase H requires only a short hybrid region, it is not possible to increase specificity by increasing the length of the ODN. In fact, increasing the length beyond the minimum is likely to have the opposite effect, by stabilizing binding to mismatched sequences, as illustrated in Fig. 1.

Based on studies performed in *Xenopus* oocytes, Woolf and co-workers concluded that it is probably not possible to obtain cleavage of an intended target RNA without also causing at least partial destruction of many non-targeted RNAs (Ref. 35). The ratio of intended to unintended hits will depend on a complex and unpredictable combination of factors that determine whether the antisense molecule and the potential targets co-localize and

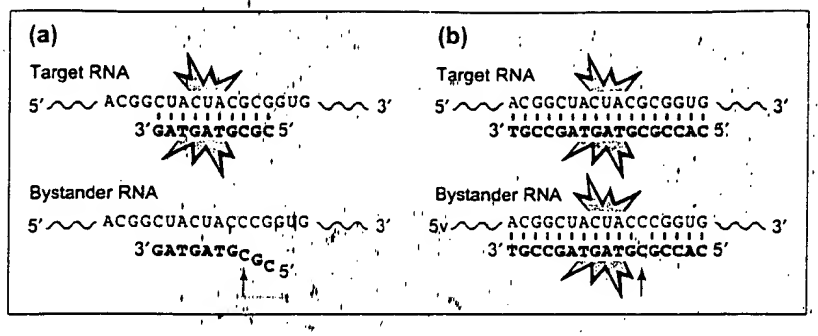

Figure 1

More is not always better. **(a)** A relatively short antisense ODN causes destruction of its intended target RNA but not a bystander RNA. This discrimination is possible because the ODN does not form enough base pairs with the bystander RNA to promote stable binding and RNase H-mediated cleavage. **(b)** A longer ODN annihilates both the target and the bystander, indiscriminately. From the standpoint of the gene hunter, an unfortunate situation exists. In general, an ODN short enough to discriminate between an RNA containing a perfect match and an RNA containing a one-base mismatch is so short that its perfect complement occurs in many different RNAs in a human cell. Thus, although it can distinguish between perfect and imperfect matches, the ODN cannot selectively destroy its target RNA. To overcome this problem, the second generation of ODNs will need special design features to enhance their specificity. In the diagrams, the 'explosion' denotes RNA cleavage by RNase H, ODNs are presented in boldface type, and sequences complementary to all or part of the ODN appear in regular lettering with the remainders of the target and bystander RNAs depicted by wavy lines; black arrows identify a nucleotide mismatch between the bystander RNA and the ODN (the bystander and the target RNA differ at this position).

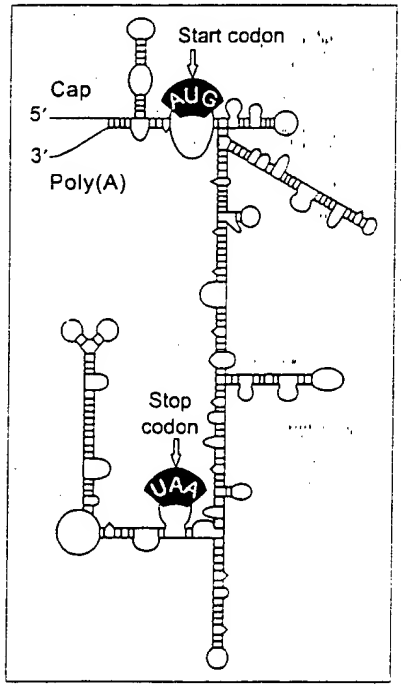

Figure 2

As illustrated by this secondary structure map of mouse β-globin mRNA, RNA molecules have an intricate array of intramolecular Watson–Crick bonds, which greatly diminish the portion of the molecule available for binding to antisense compounds and ribozymes. The positions of base pairs were determined by treating globin mRNA with structure-sensitive nucleases *in vitro*. Redrawn from Ref. 40, with kind permission.

whether the complementary sites in the RNAs are buried under proteins or are involved in intramolecular bonds that make them inaccessible. In the future, even as improvements in antisense chemistry reduce oligonucleotide binding to proteins, the specificity limits imposed by RNase H will remain and will be important to keep in mind when evaluating antisense strategies.

Target site recognition by bioengineered ribozymes is determined by Watson–Crick base pairing and thus has limits of specificity similar to those of ODNs. Ribozymes bind to their target RNAs through a recognition sequence of variable length. Somewhat counterintuitively, a ribozyme with the potential to form a larger number of base pairs with its target RNA does not necessarily have a greater power to discriminate between its intended target and a related bystander RNA than a ribozyme with a shorter recognition sequence. In fact, extending the length of the recognition sequence may reduce a ribozyme's ability to discriminate[37]. It remains to be determined whether there are recognition sequence lengths that are both short enough to allow RNAs that differ from the target at a single nucleotide to be spared cleavage and long enough to allow a unique RNA to be selectively destroyed[38]. It will not be surprising if bioengineered ribozymes are incapable of knocking out single genes, as contemplated by Bertrand and co-workers[7]. Most of these molecules are derived from either hammerhead or hairpin ribozymes[13]. In their natural setting, these ribozymes are covalently attached to their cleavage sites. They self-cleave precursor molecules of subviral (viroid) pathogens[39]. To fulfill their duties, these ribozymes have only to select their target site from the limited number of choices available in the same (small) RNA molecule. Thus, in terms of specificity, bioengineered ribozymes are expected to outperform their natural counterparts. Of course, besides binding to unintended RNAs through Watson–Crick and/or non-Watson–Crick interactions, ribozymes, like other RNAs, are highly charged molecules and have the potential to bind to cellular proteins, thereby producing biologically significant (non-antisense) effects.

As regards the theoretical limits of antisense specificity, it is important to remember that the genome is not a 'random sequence'. Sequences that constitute 'good' antisense targets in one RNA may occur in other RNAs at a higher or lower frequency than random chance would predict. One anecdote reveals how the redundancy of biological sequences could plague antisense methods. A conserved 350-base region at the 5′ end of the hepatitis C virus is considered to be a potential target for antisense drugs. This short region contains a particular 10-mer that is also present in 62 known human mRNAs (Ref. 25), and it contains two 17-mers that occur in known human DNA sequences. Ultimately, the tendency for biological sequences to be reused may limit the specificity of strategies that rely solely on Watson–Crick base pairing for recognition. This tendency will become amenable to detailed analysis soon, as more complete data about human gene sequences become available.

The three As of antisense-mediated gene ablation: access, access and access

Inside cells, it is obviously not possible to improve specificity by raising the temperature or changing the ionic strength, manipulations that are commonly used to reduce background binding in nucleic acid hybridization experiments *in vitro*. Thus, alternative strategies are needed to enhance specificity within cells. One approach has been to deploy multiple antisense compounds, each directed against a different site in the same target RNA and thereby achieve annihilation by molecular triangulation. In addition, successful efforts have been made to exploit the fact that not all

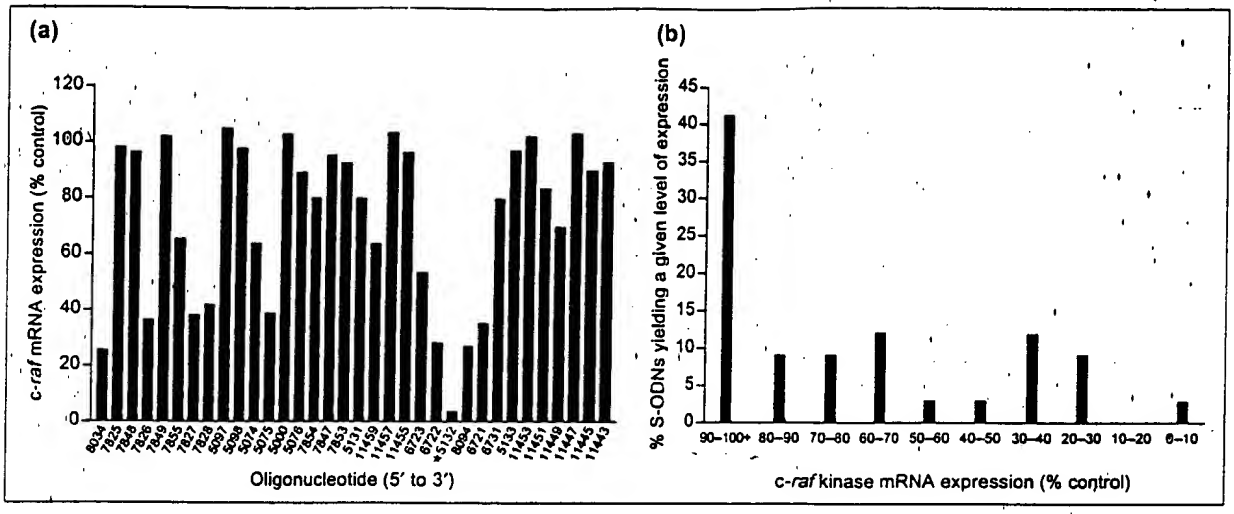

Figure 3

Superior S-ODNs can be found, but they are in the distinct minority. **(a)** Northern hybridization analysis revealed that, of 34 S-ODNs applied to A549 lung carcinoma cells, only one (5132, marked by an asterisk) caused a greater than fivefold reduction in the level of the target, c-*raf* kinase mRNA. Redrawn from Ref. 42, with kind permission. **(b)** Treatment with the majority of the S-ODNs had minimal effect and resulted in levels of the target mRNA that were 50% or more of the level in control cells.

portions of an RNA molecule are equally exposed. If a 10-mer complementary to an antisense ODN occurs in an accessible site in a target RNA and in a protected portion of a bystander, the target will be preferentially destroyed. The challenge is to identify antisense molecules that are complementary to vulnerable sites in target RNAs. This is hard to do. RNAs are complex molecules with intricate internal structures[40], as illustrated by the diagram of β-globin mRNA (Fig. 2).

Recent studies emphasize the extent to which native RNA structure restricts the binding of ODNs. Milner and co-workers[41] tested the ability of 1938 ODNs (ranging in length from monomers to 17-mers) to bind to a 122-nucleotide RNA representing the 5′ end of β-globin mRNA. They found that 'surprisingly few' ODNs bound stably to the mRNA, and concluded that binding is probably 'confined to those regions in the RNA which provide an accessible substructure'[41]. Using short (7 and 8 nucleotides) antisense molecules modified with C-5 propyne pyrimidine and phosphorothioate internucleotide linkages, Wagner and co-workers[36] also determined that the structure of the target RNA is a 'major determinant of specificity'.

Because it is very difficult to predict what portions of an RNA molecule will be accessible *in vivo*, effective antisense molecules must be found empirically by screening a large number of candidates for their ability to act inside cells. Monia and co-workers used northern hybridization to screen 34 20-nt long S-ODNs complementary to c-*raf* kinase and found only one that yielded a greater than fivefold reduction in the target mRNA (Fig. 3a; Ref. 42). Thus, only 3% of the antisense molecules tested in this system were highly effective (Fig. 3b); 40% had almost no effect[42].

Like those of ODNs, ribozyme target sites also vary in their accessibility. Chen and co-workers[43] directly demonstrated that cellular proteins and ribonucleoprotein complexes, such as ribosomes, can prevent ribozyme-mediated cleavage. They showed that a reporter gene was ribozyme-insensitive in wild-type *Escherichia coli* but was ribozyme-sensitive in a 'slow ribosome' mutant. In an accompanying editorial, John Burke (University of Vermont) remarked, 'The simple picture of ribozymes diffusing to, binding, and then cleaving an unstructured RNA is hopelessly oversimplistic'[44].

Rational and irrational design strategies are converging

At any one moment, a combination of the inherent structure of the RNA and its collection of bound proteins limits the number of accessible sites on RNA molecules, thereby providing a basis for specificity. Binding is the rare exception rather than the rule, and antisense molecules are excluded from most complementary sites (see Fig. 4). Since accessibility cannot be predicted, rational design of antisense molecules is not possible. Because design rules are lacking, effective antisense molecules are typically selected from 20–50 candidates in a time-consuming and expensive process that promises to become even more elaborate. If tests of 50 molecules identify good candidates, tests of thousands of compounds should identify better ones. If thousands are to be tested, how should they be designed? Should their sequences be based solely on their potential to form a linear series of Watson–Crick base pairs with the target, or should nucleation sites be included, as they are in naturally occurring antisense RNAs (Ref. 45)? What about non-canonical base-pair interactions, and structural features such stem loops?

The relationship between accessibility to ODN binding *in vitro* and vulnerability to ODN-mediated antisense inhibition *in vivo* is beginning to be explored, and will continue to be an active area of research in the future. It is not yet clear whether *in vitro* screening techniques of the sort used by Milner and co-workers[41] will identify ODNs that are effective *in vivo*. With so many possible sequences to choose from, and the likelihood that *in vitro* studies will not always predict *in vivo* efficacy, straightforward new screening techniques need to be developed for use in cells.

Conclusions

The original concept that ODNs and ribozymes are exquisitely specific and easy to design has been jolted by the discovery of numerous mechanisms of action, leading to non-antisense effects, and the finding that most Watson–Crick binding sites in intended target RNAs

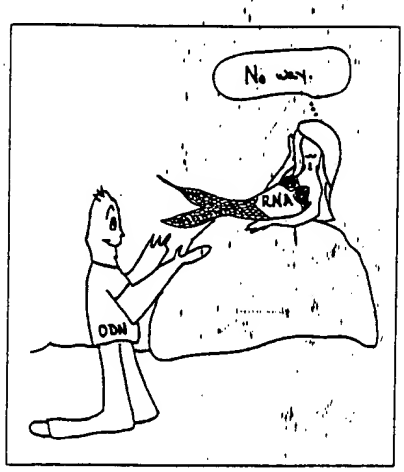

Figure 4
The structure of most potential target sites makes them inaccessible to antisense molecules and ribozymes.

are inaccessible. The time and expense necessary to screen large numbers of potential antisense molecules and ribozymes, and to carefully monitor their *in vivo* effects, raise the stakes for those seeking to use them as genetic probes. Although questions of their ultimate specificity remain, there is growing evidence that antisense molecules can be useful pharmacological tools when applied carefully[17]. In addition, certain non-antisense effects promise to be valuable therapeutically and will be fascinating to investigate. Because non-antisense effects are not currently predictable, rules for rational design cannot be applied to the production of non-antisense drugs. These effects must be explored on a case-by-case basis.

Acknowledgements

I thank J. L. Walewski and D. D. Stump (Mount Sinai School of Medicine), N. V. Bergasa (Beth Israel Medical Center), K. K. Willis (Academic Press), A. M. Krieg (University of Iowa), C. A. Stein (Columbia University), and C. F. Bennett (Isis Pharmaceuticals) for insights and T. Lefkowitz for assistance. This work has been supported by the NIDDK (grant PO1DK50795, project 2, and grant RO1DK52071) and the Liver Transplantation Research Fund (Department of Surgery, Mount Sinai School of Medicine).

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Cancer and Metastasis Reviews 17: 169–176.
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Antisense comes of age

W. Michael Flanagan
Department of Cell Biology, Gilead Sciences, USA

Key words: antisense therapeutics, phosphorothioate oligonucleotides, anti-cancer agents, protein kinase C, C-raf, Ha-ras, bcl-2

Abstract

During the last ten years, antisense technology has experienced growing pains not unlike those of adolescence. In 1992, antisense was trumpeted as one of the top 10 emerging research areas. However, 3 years later, researchers were confronted with significant problems associated with antisense oligonucleotides ranging from sequence-dependent, non-antisense effects *in vitro* to dose-limiting toxicities in preclinical models [1–3]. Many researchers had doubts whether sequence-specific antisense even existed or whether it would ever exist as a therapeutic strategy [4]. Despite these gloomy predictions, many of the challenges facing the development of antisense-based drugs as therapeutics have been overcome as evidenced by the progress of several antisense oligonucleotides in the clinic for the treatment of cancer.

Antisense technology

The concept of antisense technology is elegantly simple (Figure 1). Antisense oligonucleotides (ONs) are short (7 to 30 nucleotides) stretches of nucleic acids, usually DNA or modified DNA, that are complementary to a target messenger RNA (mRNA). The short ON selectively hybridizes to its cognate RNA by Watson-Crick base pairing rules. This RNA-ON hybrid interferes with the expression of the encoded protein by several different mechanisms including blocking RNA transport, splicing, and translation. In most cases, the RNA-ON heteroduplex forms a substrate for RNAse H, a cellular enzyme that recognizes the RNA-ON hybrid and selectively destroys the RNA portion of the hybrid. Because antisense ONs are based on the unique sequence of a gene that is implicated in the disease state, they hold the promise of being highly specific, efficacious, and less toxic than any previously developed anticancer compounds [5].

While the idea of antisense technology is simple, the development of antisense oligonucleotides as broadly applicable therapeutics has been slow and arduous [6, 7]. Like any evolving technology, antisense therapeutics have had to overcome several major barriers. First, natural oligonucleotides containing phosphodiester linkages are readily degraded in serum and fail to demonstrate any specific antisense activity *in vitro* or *in vivo* [8]. The solution to this problem was the replacement of the phosphodiester backbone linkage by a phosphorothioate backbone, which created a nuclease resistant, RNAse H competent oligonucleotide (Figure 2). With this simple modification, it became possible to demonstrate specific antisense inhibition using antisense ONs. Today, phosphorothioate oligonucleotides are the most widely used and extensively studied ONs in antisense research.

Second, not all sites on the target RNA are amenable to hybridization with an antisense ON. RNA secondary structure, which leaves only small stretches of sequence available for heteroduplex formation, affects the activity of antisense ONs [9]. For example, only one of 34 phosphorothioate ONs targeting human c-raf demonstrated potent anti-

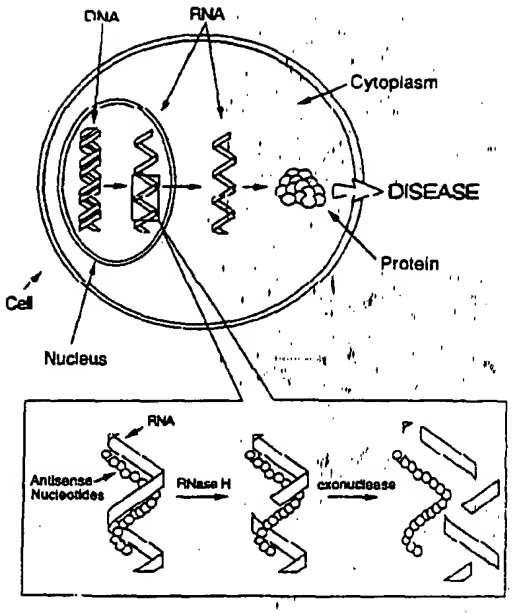

Figure 1. Mechanism of action of antisense oligonucleotides. Antisense oligonucleotides (ONs) are short, single-stranded, synthetic nucleic acids that selectively hybridize to their target RNA. In most cases, the nucleus is the active site for antisense inhibition. In the nucleus, the RNA-ON heteroduplex forms a substrate for RNase H, an enzyme that recognizes and selectively destroys the RNA portion of the RNA-ON hybrid. The allure of antisense technology is that antisense ONs can be rationally designed (based on the partial sequence of the gene) and rapidly synthesized to target any gene of interest in the entire human genome. Modified from Ref. 5.

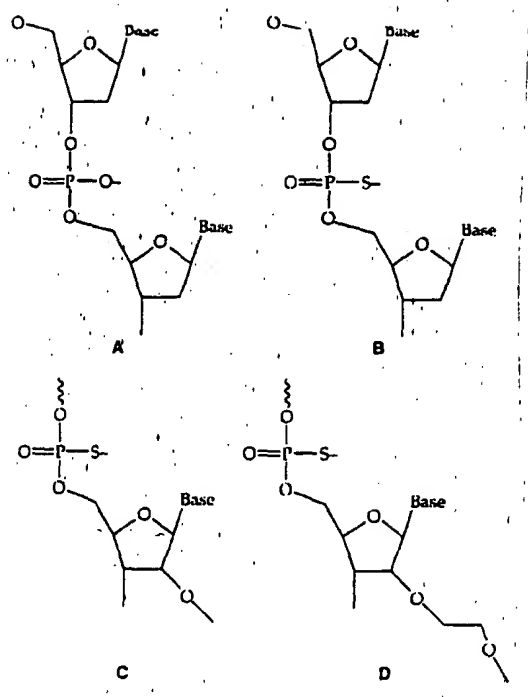

Figure 2. Oligonucleotide structure and chemical modifications. Structure of a phosphodiester (a), and phosphorothioate (b) internucleotide linkages. Modifications of the 2'-hydroxyl of the ribose sugar include: 2'-methoxy (c) and 2'-methoxyethoxy (d). Antisense oligonucleotides containing phosphorothioate backbone linkages show enhanced nuclease stability *in vitro* and *in vivo*. Substitutions of the ribose sugar at the 2'-position further enhance the stability and affinity of antisense oligonucleotides for their target RNA.

sense activity [10]. This problem can be at least partially overcome by using ONs that have enhanced binding affinity for their RNA target, such as C-5 propynylpyrimidine-modified ONs that can destabilize some secondary RNA structures [11–13]. These modified phosphorothioate ONs demonstrate higher success rates in identifying active ONs and more potent antisense effects than unmodified phosphorothioate ON [14, 15]. Recently, several reports have attempted to develop simple rules to predict antisense accessible regions of the targeted RNA [9, 16, 17], yet the most effective strategy for identifying an active ON is to empirically select and evaluate several ONs in a cell-culture based assay.

Third, phosphorothioate ONs demonstrate poor cellular permeation and lack any antisense-specific activity when added directly to cells by the cellular growth media. However, when these same phosphorothioate ONs are delivered to cells using cationic lipids that enhance cellular delivery of ONs, they demonstrated highly potent and specific antisense-dependent inhibition of their targeted proteins [18, 19]. Other nucleic acid delivery methods such as microinjection [20] and electroporation can also be used to effectively deliver biologically active antisense ONs to cells [21].

Although direct application of phosphorothioate ONs *in vitro* has not proven to be an effective delivery method, phosphorothioate ONs administered intravenously to murine tumor xenograft [10, 22, 23], transplant [24], and inflammation models have demonstrated potent and specific antisense inhibition without the need of any delivery reagent.

These surprising and provocative results have helped to revive antisense technology and have paved the way for human testing. In the cancer arena, antisense ONs targeting protein kinase C alpha (PKC-α), c-raf, Ha-ras, and bcl-2 have entered the clinic (Table 1). The status of these trials is discussed below.

Antisense ON in clinical trials for the treatment of cancer

Protein kinase C-α

Protein kinase C-α (PKC-α) is a member of a family of homologous serine/threonine protein kinases involved in cellular proliferation and differentiation. Overexpression of PKC-α has been associated with several human malignancies including breast, colon, and brain tumors [22, 25].

Inhibition of human PKC-α expression has been demonstrated using antisense ONs both *in vitro* and *in vivo* [22, 23, 26, 27]. A 20 nucleotide (nt) long phosphorothioate ON targeting the 3' untranslated region of PKC-α (ISIS3521) has been evaluated by ISIS Pharmaceuticals (Carlsbad, CA) and Novartis (Basel, Switzerland) in several human tumor cell lines grown in athymic mice. When administered intravenously once a day for 14 days, ISIS3521 suppressed tumor growth in T-24 bladder carcinoma, A549 non-small cell lung carcinoma, and Colo 205 colon carcinoma xenograft models with an 50% inhibitory dose (ID50, a dose where tumor growth is inhibited by 50% compared to a saline treated tumor-bearing animal) between 0.06 and 0.6 mg/kg/day [23]. Based on these data, ISIS3521 entered phase I clinical trials.

Two phase I trials to evaluate the safety of ISIS3521 have been completed (Table 1). In one trial, 17 patients with a variety of cancers refractory to standard cancer chemotherapy were administered ISIS3521 at a dosage ranging from 0.15 to 6.0 mg/kg/day by continuous i.v. infusion for 21 days, every four weeks. ISIS3521 was well tolerated, although some patients experienced dose dependent, drug related side-effects including thrombocytopenia and fatigue [22]. Clinical responses were observed in 3 out of 17 treated patients with all three responding patients having ovarian cancer. One patient with ovarian cancer failed to respond to treatment. The clinical responses ranged from partial remission for 11 months to minor decreases in CA-125, an ovarian tumor marker. In the second trial, 36 patients received ISIS3521 by daily i.v. infusion over a 2 hour period at doses escalating up to 2.5 mg/kg for 21 days, every four weeks. In this study, one lymphoma patient experienced a 50% reduction in measurable disease and two other patients, one with lymphoma and the other with non-small cell lung cancer, did not have signs of disease progression during the study [28].

Phase II clinical trials of ISIS3521, in which the

Table 1. Antisense oligonucleotides in clinical trials for the treatment of cancer

Product	Company	Target	Indication	Clinical progress*			
				IND	I	II	III
ISIS 3521	ISIS Pharmaceuticals Novartis	PCK-α	Solid tumors			9/97	
ISIS 5132	ISIS Pharmaceuticals Novartis	c-raf	Solid tumors			11/97	
ISIS 2503	ISIS Pharmaceutical	Ha-ras	Solid tumors		9/97		
G3139	Genta	bcl-2	Non-Hodgkin's lymphoma				
GEM231	Hybridon	R1-α subunit protein kinase A	Solid tumors				

* The solid bars indicate completion of that phase of the trial. The dates denote when the next phase of the study was initiated.

optimal dosing and clinical efficacy of ISIS3521 will be determined, have begun (Table 1). The first study will enroll approximately 50 patients with ovarian tumors who have failed to respond to up to three rounds of chemotherapy. It is estimated that this study will take about one year to complete.

c-Raf

c-Raf is a serine/threonine kinase that is an integral part of the mitogen-activated protein kinase signaling pathway. Many growth and differentiation signals that emanate from cell surface receptors are integrated by c-Raf and transmitted by phosphorylation to downstream targets including other serine/threonine kinases, transcription and translation factors. An activated form of c-Raf is oncogenic and has been associated with human malignancies.

A 20-nt phosphorothioate ON complementary to *c-raf* (ISIS5132) is being developed by ISIS Pharmaceuticals and Novartis (Table 1). In preclinical models, ISIS5132 demonstrated potent, sequence specific inhibition of human *c-raf* RNA in a variety of subcutaneously implanted human tumor cell lines in nude mice. Growth of the tumor xenografts was inhibited in a dose-dependent manner with an ID50 value at an ON concentration of 0.06–0.6 mg/kg when administered by i.v. injection once daily for 11 days. Mismatch ONs containing 4 or more nucleotide changes had no effect on tumor growth or *c-raf* RNA levels [10, 29].

Phase I clinical trials to determine the safety and pharmacokinetics of ISIS5132 have been completed (Table 1). Phase I results from two separate trials demonstrated that ISIS5132 was well tolerated when dosed daily up to 2.5 mg/kg for 21 days, every four weeks. Several patients also showed promising clinical responses including disease stabilization and reduction in serum tumor markers [29]. Based on these data, phase II trials have been initiated to further evaluate ISIS5132 in patients with previously treated breast, prostate, and colon cancers. In addition, phase I trials evaluating ISIS5132 in combination with approved chemotherapies are planned for 1998.

Ha-ras

The Ras family, which Ha-ras is a member, are GTP-regulated protein switches that are involved in cell morphology, growth, and differentiation. Point mutations in *ras* are found in nearly 30% of all human tumors. Mutations in *Ha-ras* are predominantly found in bladder cancers. A 20-nt phosphorothioate ON (ISIS2503) complementary to the start of translation of the *Ha-ras* mRNA has been shown to inhibit *Ha-ras* both *in vitro* and *in vivo* [30]. ISIS2503 appears to act through an antisense mechanism of action since the introduction of mismatches into ISIS2503 correlates with decreased inhibition of *Ha-ras* RNA expression. ISIS2503 is the third phosphorothioate antisense ON that ISIS Pharmaceuticals is developing for the treatment of cancer. Like the PKC-α and c-Raf antisense ONs, the safety and pharmacokinetics of ISIS2503 will be evaluated in patients with a variety of solid tumors that are refractory to standard chemotherapy. ISIS2503 will be administered by continuous i.v. infusion at 1 mg/kg/day for 14 days, every three weeks. The study will enroll approximately 30 patients and should be concluded by the end of 1998 [31].

bcl-2

bcl-2 is a member of a related multi-gene family that regulates programmed cell death. Overexpression of Bcl-2 blocks programmed cell death. In most low-grade, follicular non-Hodgkin lymphomas Bcl-2 is overexpressed and may play a role in chemoresistance.

An 18-nt phosphorothioate antisense ON complementary to *bcl-2* (G3139), being developed by Genta (San Diego, CA), has been shown to decrease Bcl-2 protein expression *in vitro*, and completely rid SCID mice of inoculated human follicular lymphoma cells [32]. Based on these results, a phase I trial in patients with relapsing non-Hogkin's lymphoma and high Bcl-2 expression was initiated (Table 1).

Nine patients, who had Bcl-2 positive non-Hogkin lymphoma, were administered G3139 at an escalating dose ranging from 4.6 mg/m2/day to 73.6

mg/m2/day as a continuous subcutaneous infusion for 14 days [33, 34]. One out of the nine patients demonstrated a complete response which is defined as disappearance of all disease. Two out of five patients were showed a decrease in Bcl-2 protein levels. These two patients also showed a decrease in circulating lymphocytes; however, neither patient showed a disease response. The only adverse event that was treatment related was local inflammation around the infusion site. While the results of the trial are encouraging in the treatment of non-Hodgkins lymphoma, the importance of the trial is that it is the first demonstration of antisense inhibition of a targeted protein in humans. G3139 is also being tested in a phase I/IIa trial for the treatment of prostate cancer.

Toxicities associated with phosphorothioate ONs

Although phosphorothioate ONs were well-tolerated in phase I clinical trials, ON-related toxicities have been observed in preclinical and clinical studies [35–38]. The major toxicities observed include prolongation of clotting times in all species tested, complement activation and related hypotension observed in monkeys [39], and immune stimulation in murine models [40]. These ON-associated toxicities, for the most part, appear to be independent of ON sequence and are likely related to the binding of phosphorothioate ONs to plasma proteins involved in the coagulation and complement cascade or to extracellular receptors involved in activating the immune system [36, 37, 40].

The alterations in clotting times and complement activation correlate closely to concentrations of ONs in the plasma. Decreasing peak plasma concentrations by dosing the ON continuously up to a dose of 2.5 mg/kg/day or dosing at a lower concentration can prevent any clinically significant changes in hematological parameters. Once the ON is cleared from the plasma, clotting times return to normal values and no cumulative effects of the ON have been seen [36, 37].

In contrast, effects on the kidney and liver have been observed with chronic phosphorothioate ON dosing in mice, rats and monkeys. The kidney and the liver are the primary sites for phosphorothioate ON distribution. In addition, ONs have a long half-life in these tissues and intact ON and metabolites can accumulate with repeated dosing. Changes in renal pathology and physiology have included proximal tubule degeneration, increases in urea nitrogen and creatinine in the serum, and proteinuria. In the liver, changes in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) have been observed with repeated dosing suggesting damage to hepatic cells [36, 37]. It should be noted that these toxicities have only been observed in preclinical models at doses that are 40–50 fold higher than those used in clinical trials.

The most serious effect of phosphorothioates in humans was observed in a 14 day phase II clinical trial in which 3 out of 9 patients with advanced HIV infections experienced a decrease in platelet counts that required dose interruption after 10 days of dosing at 3.2 mg/kg/day. In phase I trials, 250 patients were administered up to 4.4 mg/kg/day of a 25-nt phosphorothioate ON targeting HIV *gag* for eight days and no dose-limiting side-effects were observed; however, nearly 50% of the patients did have decreased platelet counts [41]. The longer term dosing of the ON from 10 to 14 days in the phase II trial, may have exacerbated the ON-related toxicities although combination of the ON with other anti-retroviral therapies, which were not present in the phase I trial, may have also been responsible for the increased toxicity that was observed. In phase I anticancer trials, described above, only one patient demonstrated ON-related side-effects that included decrease platelet counts. Second-generation antisense compounds may help to alleviate these ON-associated toxicities.

Second generation antisense ONs

Medicinal chemists have made a wide range of modifications to the heterocycle base, the ribose sugar, and the phosphate backbone of ONs to improve their potency and stability, and decrease their toxicity *in vivo* (Figure 2). Second-generation ONs that show the most promise are modifications of the

2' position of the ribose sugar with alkyl substituents [42–44].

Of the many 2'-alkyl modifications synthesized and tested, the 2'-methoxyethoxy modification demonstrates the best overall properties for antisense ONs including enhanced binding affinity and increased stability imparted to phosphodiester linkages [29]. Replacing the flanking phosphorothioate linkages of an antisense ON with 2'-methoxyethoxy phosphodiester linkages favorably alters the pharmacokinetics and toxicity profile of the antisense ON resulting in an increased therapeutic index [37].

One drawback of the 2'-methoxyethoxy substitution is that it interfers with RNase H recruitment and cleavage of the RNA portion of the heteroduplex. To circumvent this problem, chimeric ONs have been made that retain the best features of phosphorothioates (the ability to be recognized by RNase H) and that incorporate the 2' modified ribose sugar. The prototypical chimeric ON is 20-nt long containing five to seven 2'-alkyl modified bases with phosphodiester linkages on both the 5' and 3' end of the ON with 6–10 unmodified bases in the middle of the ON connected by a phosphorothioate backbone. This middle portion provides a docking and cleavage site for RNase H while the 5' and 3' ends provide stability, increased binding affinity and decrease the overall phosphorothioate content of the ON.

The first of these 2'-alkyl modified ONs to enter the clinic for the treatment of cancer is GEM231, a 18-nt chimeric phosphorothioate ON containing four 2'-methoxy modified ribose nucleotides on both ends. GEM231 targets the R1-α subunit of protein kinase A and will be used to treat solid tumors [45]. Additional second-generation ONs including 2'-methoxyethoxy modified antisense ONs should continue to enter the clinic for the treatment of cancer in the next few years.

Future prospects of ONs as anticancer therapeutics

Phase I trials have been completed for 3 of 4 phosphorothioate antisense ONs that have entered the clinic for the treatment of cancer. The ONs have been well tolerated and demonstrate an acceptable safety and pharmacokinetic profile to continue their development. However, significant challenges lie ahead for antisense technology. Several questions concerning the use of antisense ONs in treating cancer still need to be answered. Most importantly, will antisense ONs demonstrate efficacy in phase II trials? Will antisense resistance develop in cancers treated with phosphorothioates? Can antisense ONs be used in conjunction with existing chemotherapies? And will second-generation antisense ONs demonstrate improved pharmacokinetic profiles and reduced ON-associated toxicities? Despite these unanswered questions, it now appears that antisense technology, which represents a new class of drugs, has nearly reached maturity and will have an important clinical role in the treatment of cancer and other human diseases.

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Address for offprints: W.M. Flanagan, 333 Lakeside Drive, Foster City, CA 94404, USA; *e-mail:* michael_flanagan@gilead.com

Note added in proof: Just as the antisense field appeared to be gaining momentum, it has stumbled again. On March 17, 1998, ISIS Pharmaceuticals informed its stockholders and friends that Novartis, its antisense research and development partner, had uncovered data transgressions that affected several of their cancer compounds including antisense compounds that were tested in human tumor xenograft models. The ISIS Pharmaceutical antisense cancer compounds affected include those targeting *PKC-α*, *c-raf*, and *Ha-ras*. Currently, ISIS researchers are repeating some of the suspect animal studies and will revise or retract any published data related to the compounds. No safety data concerning the antisense compounds were affected by the data manipulation. It should be noted that these data transgressions should not taint the *bcl-2* antisense compound being developed by Genta and discussed in this review.

Does antisense make sense?

Using antisense oligonucleotides as highly selective pharmaceutical agents to block expression of disease-associated proteins has been a tantalizing promise for more than 20 years. Now that promise has become a reality: Isis Pharmaceuticals (Carlsbad, CA, USA) was granted an approval by the US Food and Drug Administration (FDA) on 24 August 1998, to market fomiversen sodium (Vitravene™), an antisense oligonucleotide intended for use in the treatment of AIDS patients with cytomegalovirus (CMV)-induced retinitis. Does this approval mark the beginning of a new era in which the exquisite specificity of antisense technology will be used to alter the course of many different diseases, or is the approval of Vitravene an anomaly unlikely to be readily repeated?

The concept behind antisense is simplicity itself: an oligonucleotide with sequence complimentary to a region of the mRNA for a targeted protein is introduced into a cell. The oligonucleotide binds to that mRNA through Watson–Crick base pairing to physically block its translation and synthesis of its specified protein. An oligonucleotide only 12 nucleotides in length has sufficient specificity to identify a particular mRNA, blocking, in theory, the synthesis of only the target protein encoded by that mRNA. Instead of being translated, the oligonucleotide–mRNA complex is degraded by RNase H. Potentially, it is an elegantly specific treatment for a host of diseases ranging from inflammation to cancer and from bacterial to viral infections, with little or no side effects.

Tried and failed

Despite the simplicity and promise of the concept, attempts to develop antisense drugs have met with many failures, and many, if not most, drug discovery scientists have discounted the utility of the concept because of the difficulties of getting the simple concept to work in practice. Because of these failures, several companies involved in antisense technology, including Gilead Sciences (Foster City, CA, USA) and Genta (San Diego, CA, USA), have moved their drug discovery efforts to other research areas. Hybridon (Cambridge, MA, USA) is still pursuing antisense programs with its own version of antisense treatment for CMV retinitis (Phase I) and research on an antisense drug for cancer. However, Hybridon had to cancel their program in HIV therapy, despite considerable financial investment, because of dose-related side effects. Even Isis had to drop its first antisense drug, a treatment for human papilloma virus, because the frequency of treatment needed was not consistent with a practical therapeutic.

In spite of the nagging problems that have plagued the use of antisense, Isis Pharmaceuticals was built and continues to be based upon the belief that the antisense technology will eventually work. Consequently, the approval of Vitravene was a particularly momentous event in the life of the young company: not only is it the first drug with a claim for an antisense mechanism to be approved for marketing by the FDA; it is the first product Isis has brought to market, and if it turns out to work through an antisense mechanism, it will provide validation for the therapeutic strategy upon which the entire company is based.

Special case?

Vitravene is administered to patients suffering from CMV infection by intraviteral injection once weekly during the initial stages of treatment and then every two to four weeks during maintenance. The direct injection into the site of infection circumvents many of the problems associated with the systemic administration of antisense therapeutics and was quite probably one of the major reasons that CMV infection was chosen as the first therapeutic target by the young company. Despite this somewhat special case for their first product, 'many other antisense drugs will follow in its footsteps', claims Stanley T. Crooke, the Chairman of the Board and CEO of Isis, who left SmithKline & French to found the company in 1989.

Stringent testing

Isis has several other antisense products in its pipeline that represent a more stringent test for the antisense strategy. In collaboration with Boehringer Ingelheim Pharmaceuticals (Ingelheim, Germany), Isis is testing an antisense drug for down-regulation of the adhesion protein ICAM-1 and its potential for use in the treatment of Crohns' Disease. This drug is currently in a pivotal clinical trial. They also envision that ICAM-1 will be an effective antisense target for the treatment of psoriasis (Phase II completed), rheumatoid arthritis, ulcerative colitis and tissue rejection after transplantation (all in Phase II). Other antisense drugs in various stages of clinical trials target protein kinase Ca (Phase II), c-raf (Phase II), and Ha-ras (Phase I) for the treatment of cancer, and a second-generation antisense product is under investigation for the treatment of CMV retinitis in AIDS patients (Phase I).

Research hurdles

The major problems in developing antisense drugs have been due to the instability of oligonucleotides in a world full of nucleases, and difficulties with delivery of the antisense reagents to the disease site and into the target cell. [For a review of delivery strategies see Miller, K.J. and Das, S.K. (1998) *Pharm. Sci. Tech. Today* 1, 377–386.] A considerable amount of research also had to be

1359-6446/99/$ – see front matter PII: S1359-6446(98)01282-3

invested in finding the right region on each mRNA to hybridize with an antisense oligonucleotide. Originally it was thought that hybridization of an antisense reagent to any portion of the target mRNA would suffice to block translation, but this reasoning was incorrect. Hybridization of an oligonucleotide to some portions of the mRNA sequence have no effect, while other regions provide insufficient inhibition of translation for the desired therapeutic response. In practice, it is necessary to explore dozens of different regions of the target mRNA before a hybridization site is found that allows the antisense oligonucleotide to shut down translation.

Finally, considerable controversy has arisen over the mechanism of action of antisense drugs: most of the oligonucleotides used for the antisense strategy bind nonspecifically to other molecules in the cell and appropriate control experiments were not always performed, particularly in the early days of antisense research, which led to misleading results. Even when appropriate control experiments are conducted, determining the specific mechanism of action can still be a challenge.

Nuclease resistance

Finding a way to increase resistance to nuclease activity was one of the first problems to be dealt with successfully. Substitution of a sulphur atom for one of the oxygen atoms in the phosphate backbone of the oligonucleotide to produce phosphorothioate oligonucleotide provided a molecule with a marked increase in stability in the presence of nuclease activity. Yet the modification still allows the formation of the Watson–Crick base pairs necessary for hybridization with a complimentary region of mRNA.

There is a drawback, however, to the use of phosphorothioate oligonucleotides: they have a tendency to bind nonspecifically to proteins and RNA. Serum albumin, for example, binds phosphorothioate oligonucleotides with an affinity in the range of 150 μM, which Crooke has noted is in a similar range as the binding of aspirin or penicillin to serum albumin [Crooke, S.T. *et al.* (1996) *J. Pharmacol. Exp. Ther.* 277, 923–937].

Specific binding to proteins, dependent upon the nucleotide sequence or the overall structure of the oligonucleotide, has also been observed and presents a more serious problem for the interpretation of antisense experiments. Control experiments usually use an oligonucleotide with a scrambled nucleotide sequence, which may not exhibit the same binding pattern to proteins as the therapeutic oligonucleotide, leading the investigator to conclude that the reagent is acting through an antisense mechanism, when in reality it is altering the activity of a key protein. Because of these complications, a wide range of circumstantial evidence must be collected to support an antisense mechanism of action. Such evidence includes demonstrating the loss of target mRNA on Northern blots in a time-course consistent with the dose–response curve for the activity of the antisense reagent both at the cell level and in the intact organism. Similar experiments can be performed to investigate the correlation of the rank order potency of various antisense agents and the loss of target mRNA and protein both *in vitro* and *in vivo*.

Persistent problem

Getting the antisense drug to the disease site is a problem that persists. Antisense reagents will probably always be limited to parenteral administration – they are not readily absorbed intact when administered orally. Several studies have shown, however, that upon injection by any of a variety of routes, antisense oligonucleotides have a half-life and distribution consistent with their use as therapeutic reagents. Finally, phosphorothioate oligonucleotides are taken up and broadly distributed in a wide variety of cell types in vitro, although the exact mechanism of uptake is unknown.

Remaining questions

In spite of the advances in dealing with the practical problems of antisense oligonucleotides, the approval granted to Isis by the FDA and the extensive data collected by Isis on the mechanism of action of Vitravene, questions remain surrounding the notion that Vitravene proves the case for antisense therapeutics. The administration of Vitravene directly into the eye does not test the general ability of an antisense reagent to reach a disease site upon systemic administration, and the possibility that some other unknown mechanism of action may account for the therapeutic effect still cannot be conclusively ruled out. In fact, it is known that at high doses, Vitravene has non-antisense effects and the compound may actually bind to the CMV coat proteins preventing the cellular uptake of the virus. However, direct binding of Vitravene to the coat proteins is not considered to be a mechanism of action of the drug, because the virus is believed to be transmitted in the eye by direct cell-to-cell contact without the release of free virus.

There may be reservations, but Isis' results with Vitravene are exciting and have succeeded in resurrecting the field of antisense therapeutics. If Vitravene is acting through an antisense mechanism, as Crooke asserts, and if some of the other antisense drugs in Isis' pipeline are also successful in reaching the market, then the antisense strategy, despite its many years of trials and tribulations, may yet pave the way for a new era of highly specific therapies for a wide range of diseases that currently have no specific means of intervention.

Robert W. Wallace
tel/fax: +1 212 254 3322
e-mail: RobWallace@nasw.org

CURRICULUM VITAE

Robert Walter Simons

Date and place of birth

January 28, 1945, Rockford, Illinois

Education

1980	Ph.D.	Molecular Biology and Biochemistry, University of California, Irvine
1972	B.S.	Biology and Chemistry, University of Illinois, Urbana

Present positions

1990-present	Associate Professor of Genetics, Department of Microbiology and Molecular Genetics, University of California, Los Angeles.
2000-present	Vice Chairman for Academic Affaires
1985-present	Member, Molecular Biology Institute, University of California, Los Angeles
1985-present	Member, Jonsson Comprehensive Cancer Center, University of California, Los Angeles

Previous positions

1985-1990	Assistant Professor of Genetics, Department of Microbiology and Molecular Genetics, University of California, Los Angeles.
1980-1985	Postdoctoral Fellow, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge MA (Advisor: Dr. Nancy Kleckner)
1981-1984	Tutor (Lecturer in Molecular Biology), Board of Tutors in Biochemical Sciences, Harvard University, Cambridge, MA
1977-1980	Graduate Student, Department of Molecular Biology and Biochemistry, University of California, Irvine (Advisor: Dr. William D. Nunn; deceased)
1973-1974	Lecturer, Department of Biology, University of New Mexico, Albuquerque
1972-1975	Staff Research Biochemist, A.I.D. Malarial Research Project, University of New Mexico, Albuquerque

Honors and awards

1978	Steinhaus Award for Outstanding Graduate Teacher, University of California, Irvine
1977-1980	National Research Service Award in Molecular Biology and Biochemistry
1980-1982	Postdoctoral Fellowship, Damon Runyon-Walter Winchell Cancer Fund
1982-1983	Postdoctoral Fellowship, National Institutes of Health
1983-1985	Special Fellowship, Leukemia Society of America
1986-1989	Junior Faculty Research Award, American Cancer Society
1989-present	Who's Who in the West; Who's Who American Men and Women in Science

Society memberships

American Society for Microbiology
American Society for the Advancement of Science
Genetics Society of America
RNA Society

Service

Editor, *Molecular Microbiology* (North American Editor for Molecular Genetics).
Ad Hoc Reviewer for NIH, NSF, Austrian Academy of Sciences, Austrian Science Foundation, Israel Science Foundation
Co-Organizer: NATO Advanced Research Workshop on "Posttranscriptional Control of Gene Expression: The Central Role of RNA Structure.
Instructor, NATO Advanced Research Workshop on Posttranscriptional Control of Gene Expression, Spetsai, Greece (1992 and 1994)
Judge, NSF Alliance for Minority Participation, U.C. Irvine (1995)
Councilor, Division H, American Society for Microbiology (1995-1997)
Chair-elect, Division H, American Society for Microbiology (1996-1997)

Publications

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Zyskind, J. W., et al
Serial No. : 08/971,090
Filed : November 14, 1997
Title : METHOD FOR IDENTIFYING MICROBIAL PROLIFERATION GENES

Art Unit : 1655
Examiner : Jeffrey Fredman, Ph.D.

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132

Sir:

1. I, Gordon Foulkes, Ph.D., am Executive Vice President for Research and Development at Elitra Pharmaceuticals Inc., 3510 Dunhill Street, Suite A, San Diego, CA 92121, the exclusive licensee of the above-identified patent application (hereinafter referred to as "Elitra").

2. I am an expert in the fields of molecular biology and cell biology and was an expert at the time of the invention. I was formerly Chief Technical Officer at Aurora Biosciences (San Diego, CA) and at Oncogene Sciences (now OSI Pharmaceutical, NY). Prior to that I was Head of the Laboratory of Cell Growth and Transformation at the Medical Research Council in the U.K. and a Senior Research Fellow at Massachusetts Institute of Technology with Professor David Baltimore. My resume is attached as documentation of my credentials.

3. I am also an expert in the field of drug discovery and the identification of therapeutic targets for drugs. I have been active in the biotechnology/pharmaceutical world for over 14 years, primarily focusing on methods to identify therapeutic targets and drug discovery. I have been involved in establishing and managing collaborations with 11 major pharmaceutical companies including Pfizer, Merck, Eli Lilly, Hoechst Marion Russell, American Home Products, Warner Lambert and Bristol Meyers Squibb, amongst others. I am also a co-inventor on several issued US patents on transcription factors as targets for drug discovery, see, e.g., U.S. Patent Nos. 5,976,793; 5,863,733; 5,776,502; 5,665,543; 5,580,722.

4. I have read the specification and the file history, including past and the outstanding office actions, and Applicants' responses, for the above-referenced patent application U.S. Serial No. 08/971,090, and I understand the issues presented by the Patent Office in the outstanding office action regarding the pending claims of the application (which will be referred to hereinafter as "the invention").

5. I believe that there was a long-felt need in the biotech industry and the field of targeted drug discovery for an invention such as that set forth in the pending claims. Over the last two decades, the pharmaceutical industry has moved predominantly toward usage of target directed approaches to drug discovery. Consequently, it is very important that drug targets be chosen which are meaningful. Appropriate selection of targets carries with it an expectation of novel drugs with significantly improved properties including greater efficacy, specificity and reduced potential for side effects. This expectation has generated a demand for new methods for targeted drug discovery.[1]

6. Inadequacy of old paradigms for drug discovery has also created a long-felt need for new methods for targeted drug discovery. Although the pharmaceutical industry has been involved in antibiotic development for over 50 years, the vast majority of such methods have been directed towards well-characterized proteins that are established targets for existing antibiotics. The result has been that only one new class of antibiotics has been discovered in the last 20 years. The widespread understanding for the need for a new generation of antibiotics emerged in the late 1980s with the rapid rise of bacterial strains exhibiting antibiotic drug resistance. For example, over 2 million people are now infected annually in US hospitals with various bacterial strains, with a resultant 90,000 deaths. Novel targets are urgently needed in order to develop new antibiotics with improved properties and no existing cross-resistance induced by current antibiotics. The best selling antibiotics today target the following three essential functions: protein synthesis (primarily ribosomes, e.g., macrolides), cell wall synthesis

[1] In target-directed approaches to drug discovery, targets are defined and chosen by disease-relevant biological criteria. Screens are then designed to identify compounds that alter the activities of these targets (inhibit or activate). Compounds that alter target activities in the desired ways may be investigated for their potential as drugs or to serve as structural leads for the development of such drugs via medicinal chemistry efforts.

(e.g., penicillins) and DNA replication (primarily topoisomerases, e.g., quinolones). It follows that proteins whose functions are required for essential cellular processes make good antibiotic targets because compounds that inhibit such functions should cause the cells to die or to stop growing. The industry understands and accepts that genes essential for life in microorganisms are likely to be good targets for antibiotics. The key is to find novel ways to identify new essential genes.

7. As evidence of this long-felt need, most, if not all, major pharmaceutical companies engaged in antibiotic research have now established either in-house or collaborative programs to try to identify new antibiotic targets. Examples of collaborations between pharmaceutical and biotechnology companies to find new antibiotic targets include, Pfizer with Microcide, Novartis with Cubist, Eli Lilly with Protein Design Labs, and American Home Products with Millennium. Other companies active in this area include Bristol Meyers Squibb, Abbott, Pharmacia Upjohn and Johnson and Johnson, amongst others. These corporate collaborations and research investments are also motivated by the great profit potential of a new antibiotic. Thus, very considerable resources, both in terms of people and money, have been spent for over a decade to identify new and effective targets for antibiotics, i.e., new essential genes.

8. These companies are employing a wide variety of different methods to identify new and effective targets for antibiotics. For example, their methods include the use of gene knockouts and temperature sensitive mutations. However, each of these methods has certain problems and all are very labor intensive and time consuming. Millennium for example, widely recognized as an industry-leading biotechnology genomics company, has reportedly identified 9 targets in their 3 year collaboration with American Home Products. Last year Cubist began a collaboration with Novartis to identify essential genes. Their goal is to provide Novartis with two new targets annually; this lower level of expectation necessary because of their "one target

at a time" approach.[2] However, in view of their being limited to the state of the art before the invention, companies find these low discovery rates acceptable.

9. The first suggestions of naturally occurring antisense RNAs potentially regulating gene expression in bacteria appeared as early as 1972. However, it was not until the 1980s' that antisense RNA was documented as a naturally occuring gene regulation mechanism. The first descriptions of the use of synthetic antisense nucleic acids and contrived antisense constructs for inhibiting the function of messenger RNAs appeared in 1985. However, despite the very widely recognized need for new antibiotic targets and the possible use of antisense methods to do this for over about the last fifteen years, I was unaware of any company successfully applying the use of genomically derived antisense fragments to identify essential genes in pathogens until Elitra licensed and began practicing the novel, specific approach as set forth by the invention. Indeed, I now believe that initial attempts to apply this general approach by others met with little or no success.

10. In contrast, the use of the invention's unique antisense method[3] by Elitra's scientists has led to the identification of nearly 400 essential genes in the bacterium *Staphylococcus aureus* in less than 6 months. This number is estimated to represent about 70% to 95% of all essential genes in this organism. In total, over 700 essential genes have been identified in 5 bacterial species by using the methods of the invention, to which Elitra is the exclusive licensee. These genes and their uses are the subject of multiple patent applications filed on behalf of Elitra.

11. I have led Elitra's technical marketing efforts to identify potential pharmaceutical partners, which we began just in late January, 2000. The interest by the drug industry to meet with Elitra Pharmaceuticals has been considerable, again reflecting the urgent

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[2] The Novartis/Cubist method of validating targets uses inhibitory protein aptamers specific to a given target tested one at a time.

[3] The methods practiced by Elitra's scientists are the same as the methods disclosed and claimed in the instant application, including practicing the methods of the invention in an automated environment to allow high throughput screening (the methods of the invention are readily adaptable to robotics).

need for new targets, the recognition of the success of the invention in finding new targets, and the limitations with existing methodologies. I have had substantive discussions with over 15 companies to date, with 3 more meetings with major pharmaceutical companies already scheduled for August. Most companies we present our results to in non-confidential descriptions react in disbelief, typically stating that it is "impossible to imagine how you could discover so many essential genes so quickly". However, when we present (under a confidentiality agreement) the method of the invention, they are amazed at the novelty of the invention and that we have been able to find a new antisense approach that works so much better than existing methodologies to identify essential gene drug targets. They also appreciate that a major factor in the success of the invention has been the use of large random libraries to generate those rare nucleotide fragments which, when expressed, possess functional antisense (i.e., message inhibitory) properties. As example of this appreciation is evidenced in the completion of a major collaboration/licensing arrangement with the pharmaceutical group of one of the world's largest consortiums, LG Chem,[4] in less than 4 months. Typically, a major collaboration of this type would take 12 to 18 months to conclude. We were able to accomplish this because of the high value LG Chem places on the uniqueness and potential of the invention and Elitra's results practicing the invention, these results being recognized as having an unexpected and unique degree of success. We are currently in discussions with several other major companies and hope to conclude similar collaborations in the near future.

12. Thus, a significant amount of objective considerations support the non-obviousness of the invention. In summary, although antisense was a very widely known method at the time of the invention and there was a very widely recognized need to identify new essential genes for antibiotic discovery for well over a decade, the invention's approach had not been discovered by others to my knowledge.[5] It is apparent from both the literature and multiple meetings with major companies, that literally hundreds of accomplished scientists, while working in this area to solve a similar long-felt need in the art, did not discover the invention and, when learning of the invention, were both surprised at its novelty and the success Elitra's

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4 The LG Chem (Korea) group generates over $70 billion in annual revenues.

5 Had not been practiced by others at the time of the invention.

scientists have been able to achieve practicing the invention. The interest by the drug industry to exploit the invention (by collaborating with Elitra, the exclusive licensee) has been and remains considerable. This interest attests to the urgent need for new drug targets, the recognition of the success of the invention in finding new targets, and the limitations with existing methodologies. Thus, I maintain my belief that the invention is extremely novel and non-obvious over other methods or literature papers in this field.

I hereby declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully Submitted

Date: August 8th 2000

Gordon J. Foulkes

J. Gordon Foulkes, Ph.D.

1220, Rancho Encinitas Drive;
Encinitas, CA 92024

Email: gfoulke@IBM.net
Phone: 619 - 756 - 2285
Fax: 619 - 756 - 2322

Employment

1999 – Present **Elitra Pharmaceuticals, San Diego, CA,**
Executive Vice President, Research and Development

1996 – 1998 **Aurora Biosciences Corporation, San Diego, CA**

Co-presented $40MM IPO and led marketing of drug discovery technologies resulting in $100MM in collaborative revenues from 4 major pharmaceutical companies. All technical milestones delivered ahead of schedule.

Chief Technical Officer and Member of the Board of Directors

Managed annual research budget of $38 million and directed team of over 100 scientists and engineers. Overall responsibility for scientific and technical focus, and technical marketing. Managed scientific collaborations with Merck & Co., Warner-Lambert, Eli Lilly & Company and Bristol-Myers Squibb. Responsibilities included development of the ultra-high throughput screening system (UHTSS), drug discovery assay technologies and functional genomics.

1987 – 1996 **Oncogene Science, Inc., New York, NY**

Raised over $47MM net in two secondary offerings and created technology packages leading to seven major pharmaceutical drug discovery collaborations. All collaborations successfully renewed at least one time. Several products taken from gene through IND.

1994 - 1996 **Appointed to the Office of the Chief Executive and the Board of Directors**

1992 – 1995 **Vice President and Chief Scientific Officer**

Overall responsibility for 120 scientific staff members, including 40 Ph.D.s. Annual research budgets of $14.3 million (pharmaceutical), and $4.5 million (research products and diagnostics). Managed collaborations with Pfizer, Hoechst AG, Ciba-Geigy, American Home Products, Marion Merrell Dow and Becton Dickinson. Directed drug discovery programs for 27 targets including cancer, cardiovascular disease, virology, hematopoiesis, diabetes and inflammation.

1990 - 1992 **Vice President and Director of Therapeutics**
Managed a division of 30 scientists (Ph.D.s. and technicians). Annual research budget of $4.5 million, research programs in the areas of cancer, atherosclerosis and hematopoiesis. **Pioneered high throughput screening to the pharmaceutical industry as a novel approach to drug discovery.**

1987 - 1990 **Director of Therapeutics**
Initiated the development of gene transcription and high-throughput screening as the major drug discovery focus of Oncogene. Directed the cancer research program with Pfizer ($2.5 million annually for 5 years). Team of 16 scientists.

1984 - 1987 **Tenured Member of the Scientific Staff**
The Medical Research Council (MRC), National Institute for Medical Research, London, U.K. Research focus: receptor and viral signaling mechanisms.

1982 - 1984 **Senior fellow** in the Laboratory of Dr. David Baltimore, Massachusetts Institute of Technology Massachusetts, U.S.A. Supported by a MRC fellowship. Research focus: molecular biology of oncogenes.

1980 - 1981 **Postdoctoral fellow** in the Laboratories of Dr. R. Erikson and Dr. J. Maller, University of Colorado, Denver, Colorado, U.S.A. and Dr. L. S. Jefferson, Hershey Medical Centre, Hershey, Pennsylvania, U.S.A. Research focus: the role of protein phosphorylation in hormone action and cell transformation.

Education

1979 University of Dundee, Scotland. Ph.D. Biochemistry "The Regulation of Protein Phosphatase-1 by Specific Inhibitor Proteins." Supervisor: Professor P. Cohen.

1976 University College Cardiff, Wales. B.Sc. Biochemistry. First Class Honors.

Other Achievements

Aurora Biosciences Corp.

Operations: increased technical staff from 20 to over 100 in 18 months. Designed over 40,000 sq.ft. of engineering, biology and chemistry labs. Participated in setting up human resources, internal financial systems, and project management.

Storage and Retrieval System: successfully concluded the first stage of the UHTSS, on schedule, now accepted by both BMS and Lilly. The unit stores in excess of 1.5 million compounds, and can select and deliver over 100,000 compounds per day for screening/replication including miniaturized 3,456 well assay format.

Established High Throughput Screening: 20,000 compounds per day (384 well format).

Drug Discovery Assay Technologies: Aurora assembled a broad portfolio of fluorescent assay technologies for use with a wide range of disease targets. Technologies address GPCRs, ion channels, proteases, protein-protein interactions, and transcription factors. All partners high throughput-screening assays developed on or ahead of schedule.

Oncogene Science

A major focus over the last 18 months had been on business development/strategic planning for OSI. This included the lead OSI role both in preparing the prospectus and presenting the Company's technology in a secondary stock offering. Raised $30.6 million net (Banker - Robertson Stephens).

IND Pipeline: At least one target from each of the collaborative/proprietary programs had progressed from concept stage through to in vivo active leads with committed chemistry. For example, the Pfizer collaboration filed an IND for an oncogene inhibitor in 3Q/96, while the Novartis collaboration had TGF-β3 in Phase I for oral mucositis and Phase II for wound healing.

Cosmeceuticals: Took the lead OSI research role in the development of a new alliance with Pfizer, applying molecular biology to the discovery of cosmeceuticals. Finalized 2Q/96.

Virology: Established partnership with the MRC and alliances with both MIT and Southwest Research Foundation to create an anti-viral drug discovery consortium. Initial targets include Hepatitis B and C, HIV, and influenza. This lead to two major anti-viral collaborations, one with BioChem Pharma and the other with Sankyo.

Muscle Wasting: Established consortium with Cold Spring Harbor Laboratories and the Association Francaise Contre les Myopathies (AFM) to treat muscular dystrophy. Obtained approximately $1 million in funding.

1994: Drafted and presented the scientific plan for collaborative programs with Ciba-Geigy on TGF-β3. Following successful preclinical development of this recombinant biological the drug proceeded into two Phase I/II trials with Novartis; oral mucositis and wound healing.

1993: Drafted and presented a scientific proposal for the development of new cardiovascular drugs with Marion Merrell Dow. Resulted in a $12.5 million/5 year collaboration.

Drafted and presented the scientific plan focused on transcriptional regulation of key targets in arthritis, Alzheimer's disease and bile acid metabolism to Hoechst. Resulted in a four-year collaborative program. Over $2.2 million in Small Business Grants from NIH.

1992: Drafted and presented a scientific proposal focused on gene transcription as a drug discovery approach to Wyeth Ayerst. Specific gene targets in asthma, osteoporosis, immune suppression and diabetes. Resulted in an initial two-year collaborative program, subsequently renewed and extended.

Principal Investigator on a $3 million NCDDG grant from NIH to develop drugs for CML

1991: Co-wrote scientific research plan targeting oncogenes/anti-oncogenes, as cancer diagnostic markers. Resulted in a five-year renewal of the collaboration with Becton Dickinson.

Principal role in analysis of diagnostic products/patents developed by Applied bioTechnology (Dr. Robert Weinberg's company), leading to its acquisition by OSI.

Participated in drafting and co-presented follow-on offering (Wertheim Schroeder) raising $17.1 million net.

1990: Wrote the scientific plan for the collaborative renewal program with Pfizer, targeting oncogenes/anti-oncogenes, resulting in a second 5 year contract, $3.5 million annually.

Key Academic Research Achievements

Over 50 major publications and reviews prior to joining Oncogene Science in 1987. Examples:

- Discovery and characterization of mammalian protein-tyrosine phosphatases: J. Biol. Chem. 258, 431-438; FEBS Lett. 130, 197-200.
- Discovery in transformed cells of tyrosine phosphorylated, sequence-specific DNA binding proteins: Nature 325, 552-554.
- Development of the first bacterial expression system and purification to homogeneity of a protein tyrosine kinase: J. Biol. Chem. 260, 8070-8077.
- Identification of a novel mechanism of insulin action *in vivo*. Eur. J. Biochem. 97, 251-256; J. Biol. Chem. 257, 12,493-12,496.
- Identification of serine/tyrosine protein kinase cascade systems. Proc. Natl. Acad. Sci. U.S.A. 82, 272-276; EMBO J. 4, 3173-3178; Proc. Natl. Acad. Sci. U.S.A. 84, 4408-4412.
- Identification of protein phosphatases in translational control. Proc. Natl. Acad. Sci. U.S.A. 82, 272-276; Proc. Natl. Acad. Sci. U.S.A. 79, 7091-7096; J. Biol. Chem. 258, 1439-1443.
- Discovery of a new human oncogene. Nature 325, 635-637.
- Cloning of TGF-β3. Proc. Natl. Acad. Sci. USA 85, 4715-4719.

Patents

1. TGFβ3 series
2. Methods of Trancriptionally Modulating Gene Expression series
3. Novel Approach Towards Functional Genomics in Mammalian Cells series